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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C07K 15/06, 15/14	A1	(11) International Publication Number: WO 86/ 00078
		(43) International Publication Date: 3 January 1986 (03.01.86)

(21) International Application Number: PCT/AU85/00119

(22) International Filing Date: 3 June 1985 (03.06.85)

(31) Priority Application Numbers: PG 5427
PH 0031

(32) Priority Dates: 8 June 1984 (08.06.84)
4 April 1985 (04.04.85)

(33) Priority Country: AU

(71) Applicants *(for all designated States except US)*: MONASH UNIVERSITY [AU/AU]; Wellington Road, Clayton, VIC 3168 (AU). PRINCE HENRY'S HOSPITAL [AU/AU]; St. Kilda Road, Melbourne, VIC 3000 (AU). ST. VINCENT'S INSTITUTE OF MEDICAL RESEARCH [AU/AU]; 41 Victoria Parade, Fitzroy, VIC 3065 (AU). BIOTECHNOLOGY AUSTRALIA PROPRIETARY LIMITED [AU/AU]; 28 Barcoo Street, East Roseville, NSW 2069 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : ROBERTSON, David, Mark [AU/AU]; 6 Floreat Court, Glen Waverley, VIC 3150 (AU). DE KRETSER, David, Moritz [AU/AU]; 1 Leura Street, Surrey Hills, VIC 5127 (AU). HEARN, Milton, Thomas, William [AU/AU]; 263 Union Road, Balwyn, VIC 3103 (AU). BURGER, Henry, George [AU/AU]; 36 Berry Street, East Melbourne, VIC 3002 (AU). FINDLAY, John, Kerr [AU/AU]; 3 Barloa Road, Mont Albert, VIC 3127 (AU). MORGAN, Francis, Joseph [AU/AU]; 41 Alfred Crescent, North Fitzroy, VIC 3068 (AU). FORAGE, Robert, Gregory [AU/AU]; 80 Awaba Street, Mosman, NSW 2088 (AU).

(74) Agent: CLEMENT HACK & CO.; 29th Floor, BHP House, 140 William Street, Melbourne, VIC 3000 (AU).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: INHIBIN ISOLATED FROM OVARIAN FOLLICULAR FLUID

(57) Abstract

A protein which satisfies all the biological criteria which are characteristic of inhibin has been isolated from a gonadal source. The purification and characterization of inhibin and the use of the purified material to raise antibodies, the use of inhibin and said antisera in a quantative radioimmunoassay, and applications *in vitro* and *in vivo* of inhibin and antibody to inhibin, are described. There is provided a purified protein, inhibin, characterised in that (a) the apparent molecular weight as determined by SDS-PAGE is 56,000 ± 1,000; (b) the isoelectric point is in the range 6.9 - 7.3; (c) the protein can bind specifically to Concanavalin A-Sepharose; (d) the protein consists of two sub-units, characterised in that (i) their apparent molecular weights as determined by SDS-PAGE are 44,000 ± 3,000 and 14,000 ± 2,000 respectively; (ii) the isoelectric point of the 44,000 molecular weight sub-unit is in the range 6.0 - 7.0; (iii) the N-terminal amino acid sequences of the two sub-units are as described herein; (e) the protein can suppress follicle stimulating hormone,(FSH) but not luteinising hormone (LH) thyroid stimulating hormone or prolactin in an *in vitro* bioassay system; (f) the protein can be labeled with radioactive iodine. There is also provided a method for isolating and purifying inhibin from mammalian ovarian follicular fluid, characterised by: (a) one or more gel permeation chromatography steps; (b) one or more reversed-phase high performance liquid chromatography steps; (c) one or more preparative polyacrylamide gel electrophoresis steps; (d) Electrophoretic elution of the purified inhibin.

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INHIBIN ISOLATED FROM OVARIAN FOLLICULAR FLUID

This invention relates to the isolation of a biologically active factor from mammalian ovarian follicular fluid, and valuable uses of that factor.

On the basis of circumstantial evidence and limited experimentation it was suggested as early as 1932 that the gonads produced a non-steroidal factor, termed inhibin, which was capable of selectively suppressing the pituitary gland secretion of follicle stimulating hormone (FSH) (McCullagh, Science 76, (1932) 19). Since that time the development of radioimmunoassays to measure FSH has led to the accumulation

of a substantial body of evidence to suggest that inhibin exists, but it was not until the early 1970's that any attempt to isolate and identify this substance was made. Since that time a number of groups of investigators have attempted to purify inhibin from a several gonadal sources with conflicting results (de Jong, Mol. Cell. Endocrinol. 13, (1979) 1). Some investigators have claimed to have isolated and sequenced inhibin from human seminal plasma, with the molecular weights of their species of inhibin being 5,000 and 14,000 daltons (Seidah et al., FEBS Letters 167, (1984) 98; Sheth et al., FEBS Letters, 165, (1984) 11). Furthermore, the gonadal origin of these material has been seriously questioned (Beksac et al., Int. J. Andrology 7, (1984) 389; Lilja and Jeppsson, FEBS Letters 182, (1985) 181). Other groups of investigators have utilized fluid collected from the seminiferous tubules of the testis (rete testis fluid) and also ovarian follicular fluid to attempt to isolate gonadal inhibin. As yet these attempts, despite being carried out over a period of 12 years, have been unsuccessful in obtaining a purified material. This background indicates that there is no general agreement as to the nature, chemical features or site of production of the substance defined as inhibin.

The properties of bovine follicular fluid extracts have led to the postulate that there is a substance or substances, "inhibin", with specific functions. We have now isolated a material from a gonadal source which satisfies all the biological criteria which are characteristic of inhibin.

The present invention relates to the purification and characterization of inhibin and to the use of the purified material to raise antibodies, the use of inhibin and said antisera in a quantative radioimmunoassay, and applications in vitro and in vivo of inhibin and antibody to inhibin.

According to one aspect of the present invention there is provided a purified protein, inhibin, characterized in that

a) the apparent molecular weight as determined by SDS-PAGE is 56,000 ± 1,000

b) the isoelectric point is in the range 6.9-7.3

c) the protein can bind specifically to Concanavalin A-Sepharose

d) the protein consists of two sub-units, characterized in that

(i) their apparent molecular weights as determined by SDS-PAGE are 44,000 ± 3,000 and 14,000 ± 2,000 respectively

(ii) the isoelectric point of the 44,000 molecular weight sub-unit is in the range 6.0-7.0

(iii) the N-terminal amino acid sequences of the two sub-units are as described hereinbelow

e) the protein can suppress follicle stimulating hormone (FSH) but not luteinising hormone (LH), thyroid stimulating hormone or prolactin in an in vitro bioassay system

f) the protein can be labeled with radioactive iodine.

According to another aspect of the invention, there is provided a method for isolating and purifying inhibin from mammalian ovarian follicular fluid, characterized by the following steps:

a) one or more gel permeation chromatography steps;

b) one or more reversed-phase high performance liquid chromatography steps;

c) one or more preparative polyacrylamide gel electrophoresis steps;

d) Electrophoretic elution of the purified inhibin.

Preferably the gel permeation chromatography step is carried out using a gel permeation support with high pore volume, for example Sephacryl S200 or Sephadex G100 (both Sephacryl and Sephadex are trade marks of Pharmacia). Preferred elution methods utilize volatile solvents

containing, for example, ammonium acetate, acetic acid, or similar compounds, to allow direct recovery of biological activity by lyophilization or vacuum drying.

Preferably the reversed-phase high-performance liquid chromatography is carried out using chemically-bonded N-alkylsilica column packings of narrow particle size distribution, most suitably 5-10 μ. The eluents used may be volatile or non-volatile, and contain ionic modifiers such as trifluoroacetic acid (TFA), ammonium bicarbonate, ammonium acetate, or sodium phosphate, in a gradient of water with a miscible organic solvent such as methanol, acetonitrile, or isopropanol. A preferred procedure utilizes a gradient of 0-50% acetonitrile in 0.1% TFA. Various preparative polyacrylamide gel electrophoresis (PAGE) methods can be employed in the presence of sodium dodecyl sulphate (SDS) using PAGE gels of various porosities and cross-linking content. A preferred buffer system for electrophoresis is based on the method of Laemmli, as described in Nature 227, (1970) 680.

There is further provided a method for raising specific antibodies to the inhibin, said antibodies having the ability to neutralize the activity of inhibin in the in vitro bioassay, and to cause an increase in gonadal weight in vivo.

There is still further provided a method for the radioimmunoassay of inhibin which can be used for the measurement of inhibin in biological samples such as plasma, serum or urine.

One embodiment of the present invention will now be described in detail by way of example only with reference to the following non-limiting examples, and the accompanying drawings in which:

Figure 1 shows the elution profile of inhibin activity in bovine ovarian follicular fluid (bFF) fractionated on Sephacryl S-200.

Figure 2 shows the elution profile of inhibin activity in bFF fractionated on Sephadex G100.

Figure 3 shows the elution profile of inhibin activity in bFF fractionated by reversed-phase high performance liquid chromatography (RP-HPLC) after prior gel chromatography.

Figure 4 shows reduced and non-reduced SDS-polyacrylamide gel electrophoretic patterns of four sequential fractions obtained by preparative PAGE.

Abbreviations used herein are as follows:

bFF	- bovine ovarian follicular fluid
RP-HPLC	- reversed-phase high performance liquid chromatography
PAGE	- polyacrylamide gel electrophoresis
FSH	- follicle stimulating hormone
LH	- luteinising hormone
U	- units
GF	- gel filtration
kD	- kilo Daltons
TFA	- trifluoroacetic acid
SDS	- sodium dodecyl sulphate

Example 1: Purification of inhibin from bovine ovarian follicular fluid

The purification procedure is based on the sequential application of one or more gel permeation steps, one or more reversed phase high resolution chromatography steps and one or more PAGE steps.

Collection of bovine follicular fluid (bFF)

Bovine ovaries were obtained from local abattoirs and bFF aspirated into a chilled vessel containing the protease inhibitors Trasylol (10 U/ml) and phenylmethylsulphonyl fluoride (24 µg/ml). The bFF was stored frozen at -20°C.

The procedure for purification of inhibin consisted of 4 stages or steps. In the sections below an outline of the procedure is described and details of the purification procedure are presented.

The steps in the purifications procedure are as follows:

(A) Gel permeation chromatography on Sephacryl S-200.

(B) Gel permeation chromatography on Sephadex G-100.

(C) Reversed phase high performance liquid chromatography.

(D) Preparative polyacrylamide gel electrophoresis.

(E) Electrophoretic elution of samples.

The purification of inhibin was monitored by the bioassay method of Scott et al. (Endocrinology 107, (1980) 1536), used with minor modifications (Au et al., Endocrinology 112, (1983) 239). The method is based on the ability of inhibin to cause dose-dependent reduction of FSH but not LH cellular content of rat anterior pituitary cells in culture.

Step A:

Gel Permeation Chromatography on Sephacryl S200: Elution Buffer 0.05M Ammonium Acetate pH 7.0

bFF (50-100ml) was diluted with 0.05M ammonium acetate pH 7.0 (25-50 ml) and centrifuged (12000 g x 30 min at 4°C). The supernatant (75-150 ml) was fractionated on a Sephacryl S200 gel filtration column (9 x 90 cm) at a flow rate of 70-100 ml per hour. As seen in Fig. 1, inhibin activity was located in a void volume region (MW $\geq$ 90,000) of this column. 90% recoveries of inhibin activity were obtained with a 3-4 fold increase in specific activity.

Step B:

Gel Permeation Chromatography on Sephadex G100: Elution Buffer 4M Acetic Acid

Void volume fractions (A, B, C and D, Fig. 1) were combined and 25-50% of the pooled fractions were acidified with glacial acetic acid (chromogen-free) to a final concentration of 4M acetic acid and kept at 4°C for one hour. The remaining 50-75% was stored frozen at -20°C prior to subsequent fractionation. The acidified pool (approximately 120 ml) was applied to a Sephadex G100 gel filtration column (9 x 90 cm) with 4M acetic acid as eluting buffer at a flow rate of 70-100 ml per hour. All operations with both gel filtration columns were performed at 4°C. Under these conditions the bulk of the inhibin activity eluted in a lower molecular weight region (elution volume 1760-1880ml, MW range 20,000-60,000, Fig. 2) with a 10-20 fold increase in specific activity and 45% recovery of inhibin activity in this region. Using analytical columns (e.g., 2.5 x 100 cm) similar profiles of activity with higher specific activities (10-50 fold) have been observed.

Step C:

Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

The column fractions (elution volume approximately 1760-1910 ml) from Step B were pooled prior to loading onto the RP-HPLC column. The column employed was an Ultrapore RPSC (Beckman, Berkeley, California). The mobile phase used was a linear gradient between 0.1% TFA in water and 50% acetonitrile in 0.1% TFA; the flow rate was 1 ml/per minute and 0.5 ml fractions were collected. Three loading procedures were employed: (a) the sample was lyophilised and 1 mg dissolved in 4M acetic acid to a concentration of 8-10 mg dry weight/ml, centrifuged in approximately 100μl 4M acetic acid and applied to the HPLC column via the injector; (b) the lyophilised

material (5-10 mg) was dissolved in 20 ml of 4M acetic acid, centrifuged and loaded onto the column via a solvent port on the HPLC; (c) the unlyophilised material (approximately 100ml) was filtered through a 0.5 μm filter (FH; Millipore Corp) prior to loading via a solvent port on to the column at a flow rate of 2 ml/minute.

The inhibin regions from the bulk runs (b) and (c) were rechromatographed. Each fraction was combined with the contents of the corresponding tubes from the repeat runs (if required) and aliquots were taken for bioassay, amino acid analysis and SDS-PAGE. The acetonitrile from each fraction was then removed by evaporation under N_2 and the sample lyophilised. As seen in Fig. 3, inhibin activity was found in one region of the chromatogram, corresponding to approximately 30% acetonitrile. The sample load in this experiment was 1 mg.

Recoveries of inhibin of 40% were obtained with the various loading procedures, although the HPLC column performance was markedly influenced by the latter two procedures. A 10-fold increase in specific activity was attained with this HPLC step, with an overall 160-fold increase in specific activity.

Step D:

Preparative Polyacrylamide Gel Electrophoresis

The inhibin-containing fractions obtained by RP-HPLC were dissolved in non-reducing sample buffer (0.06 M Tris-HCl pH 6.8, 12.5% glycerol, 1.25% w/v SDS and 0.006% bromophenol blue) and fractionated on a vertical polyacrylamide gel electrophoresis apparatus (Reid and Bieleski, Analytical Biochemistry, 22, (1968) 374) with modifications. The SDS polyacrylamide gel electrophoresis solutions (Laemmli, Nature 227 (1970) 680) consisted of a stacking gel (0.125M Tris-HCl pH 6.8, 0.1% w/v SDS, 5% acrylamide, 0.13% Bis acrylamide, 0.1% ammonium persulphate) and a separating gel (0.38M

Tris-HCl pH 8.8, 0.1% w/v SDS, 7.5% acrylamide, 0.2% Bis acrylamide, 0.03% ammonium persulphate). The electrophoretic buffer was 2.5mM Tris-glycine buffer containing 0.05% (w/v) SDS. The protein load (500-700 μg) was divided between the eight sample slots.

The gels were electrophoresed initially at 20mA until the sample had migrated into the separating gel (1.5h), then the current was increased to 30mA for the duration of the run (4h) until the bromophenol blue marker reached the bottom of the gel. The gel was stained with 0.5% Coomassie blue in acetic acid: isopropyl alcohol:water 1:3:6 v/v (15 min) and destained with acetic acid: methanol:water, 50:165:785 v/v, and the inhibin region (molecular weight approximately 56,000) which was visualised by this procedure was sectioned into 2 mm slices using a scalpel and ruler. Gel slices above and below the inhibin region were also taken. The gel slices were stored in sealed tubes at -15 to -20^{o}C prior to electrophoretic elution.

Figure 4 shows reduced and non-reduced SDS-gel electrophoretic patterns of 4 sequential fractions (A, B, C and D) obtained by preparative PAGE purification. Inhibin activity was located primarily in fraction B (apparent molecular weight 56,000 $\pm$ 1,000 mean $\pm$ SD; 5 purified inhibin preparations). Under reducing conditions, fraction B reduced into two major bands with apparent molecular weights of 44,000 $\pm$ 3,000 and 14,000 + 2,000 (n = 5) (Lane E). The Laemmli (1970) SDS-PAGE system was employed. Proteins were localized by silver staining. Protein standards used were: bovine serum albumin (molecular weight 67,000); ovalbumin (43,000); carbonic anhydrase (29,000); goose lysozyme (21,000); hen egg lysozyme (14,500). The reductant was 0.1% 2-mercaptoethanol.

Step E:

Electrophoretic Elution at Room Temperature

The method used was modified from that of Hunkapiller et al. (Methods in Enzymology, 91 (1983) 227). Gel slices were diced in distilled water with a razor blade, washed in elution buffer (0.1% SDS in 0.05M NH_4HCO_3) for 5 min and placed in an electrophoretic elution cell fitted with dialysis membrane discs (6,000-8,000 molecular weight cut off). The gel slices were covered with soaking buffer (2% SDS in 0.4M NH_4HCO_3), and overlayed with elution buffer (0.1% SDS in 0.05M NH_4HCO_3). Solid sodium thioglycollate, to a final concentration of 0.5mM, was added to the elution buffer. Gel pieces were allowed to soak for 3-5hr prior to the initiation of the electrophoretic elution process at 50V (direct current). After 12-16 hr the elution buffer was replaced with dialysis buffer (0.02% SDS in 0.01M NH_4HCO_3) followed by further electrophoretic elution at 80V (direct current) for 20-24 hr until the Coomassie blue stain and protein had migrated into the sample collection well. The eluted sample was removed from the collection well by means of a bent-tipped 50µl Hamilton syringe, aliquoted and either frozen or lyophilised. Samples of the purified fractions were set aside for the in vitro bioassay, amino acid analysis and molecular weight determination using SDS-PAGE as employed with the silver staining technique.

Using purification procedures based on the above methods, i.e. a combination of gel permeation chromatography, RP-HPLC and preparative PAGE, inhibin activity was recovered as a single protein band on SDS-PAGE (Fig. 4), with an apparent molecular weight of 56,000 ± 1,000 (5 preparations).

The purified preparations of inhibin suppress FSH but not luteinising hormone, thyroid stimulating hormone or prolactin in the in vitro bioassay, indicating that the purified product is specific in suppressing FSH. The suppression is not due to non-specific toxic effects.

Example 2: Alternative Purification Procedures

Inhibin was isolated as in Example 1, except that inhibin was precipitated by adjusting the pH of the void volume fraction from Step A to pH 4.75 with 4M acetic acid and centrifuged at 12,000 x g for 30 min at 4°C. The resulting pellet was dissolved in 0.05M ammonium acetate pH 7.0. The solubilization of the pellet was aided by homogenization and sonication in buffer at room temperature. The sample was adjusted to 4M acetic acid with glacial acetic acid and centrifuged prior to application to the column for the second gel permeation step (Step B). The overall recoveries of inhibin activity including pH precipitation and acidification of the dissolved pellet was 34%. The modification has the advantage that the column sample volume is reduced by 75% allowing a greater throughput of material. Inhibin activity was recovered in similar column fractions (elution volumes 1700-2100 ml) to that in the procedure of Example 1 following fractionation by gel filtration with 4M acetic acid as elution buffer. The subsequent behaviour of inhibin on the RP-HPLC and the PREP-PAGE procedure was not influenced by the various modifications examined in this example.

Example 3: Further Chemical Characterization of Inhibin

Analytical SDS-PAGE of the final product under non-reducing conditions gave a single band with an apparent molecular weight of 56,000 $\pm$ 1,000 (mean $\pm$ SD, 5 preparations) while under reducing conditions two major bands with apparent molecular weights of 44,000 $\pm$ 3,000 and 14,000 $\pm$ 2,000 (5 preparations) were observed. Evidence of heterogeneity was observed as assessed from electrophoresis of the 56kD band under non-reducing conditions and the 44kD band under reducing conditions. The apparent molecular weight range for the 56kD material was between 54,000 and 57,000

while the 44kD material ranged from 42,000 to 46,000. A single band was observed with the 14kD band. These findings are consistent with the glycoprotein nature of this molecule.

The pI values of intact inhibin and the larger sub-unit were determined using the 2 dimensional PAGE system of O'Farrell, P.H., J. Biol. Chem, 250 (1975), 4007-4021. Intact inhibin was detected by silver stain and showed a single band with an apparent molecular weight of 56,000 but with several closely associated spots with pI values in the pH range 6.9-7.3. These data are suggestive of a glycoprotein preparation. The 44,000 molecular weight sub-unit showed a single band with an apparent molecular weight of 46,000 with several closely associated spots with pI values in the pH range 6.0-7.0, suggesting that this sub-unit is a glycoprotein.

Further evidence that inhibin is a glycoprotein was established by:

(a) The ability of radiolabelled inhibin to bind to the lectin Concanavalin A immobilized on Sepharose (Trade Mark of Pharmacia, Uppsala, Sweden). From several experiments 15-17% of the tracer bound to the lectin and was released following elution with the sugar methyl-α-D-glucopyranoside (Calbiochem, San Diego, USA).

(b) Binding of horseradish peroxidase-labelled wheat germ lectin to inhibin, following fractionation of inhibin on SDS-PAGE and electrotransfer of the protein on to nitrocellulose. The binding of the lectin was monitored by the intensity of the peroxidase colour reaction. Lectin binding was associated with the 56kD intact protein and with the 44kD sub-unit.

Example 4: The N-Terminal Amino Acid Sequence of the Two Sub-Units of Inhibin

A purified preparation of inhibin was reduced and carboxymethylated and the two sub-units, of apparent molecular weight 44,000 and 14,000 respectively, were separated by PAGE and recovered from the gel by an electroelution process as described in Step E, Example 1 above. The SDS was removed by methanol precipitation of inhibin, and the N-terminal amino acid sequence determined.

The sequences of the two sub-units are:

Residue	44 kD Sub-Unit	14 kD Sub-Unit
1	xxx	Tyr
2	Ala	Leu
3	Val	Glu
4	Gly	yyy
5	Gly	Asp
6	Phe	Gly
7	Met	Lys
8	Arg	Val
9	Arg	Asx
10	Gly	Ile
11	Ser	Gln
12	Glu	yyy
13	Pro	Lys
14	Glu	Lys
15	Asp	
16	Gln	

xxx = ambiguous

yyy = unable to be determined - insufficient material in these experiments

Asx = Asn or Asp

Example 5: Raising of Antibodies to Purified Inhibin

19 μg of inhibin purified as described above was dissolved in 600 μl of Dulbecco's Phosphate Buffer pH 7.4 and emulsified with an equal volume of an oil-based adjuvant (for example, Marcol 52: Montanide 888 in the ratio 9:1. Marcol 52 is a Trade Mark of Esso, and Montanide 888 is a Trade Mark of S.E.P.P.I.C., Paris). Two hundred μl was injected into each of four intramuscular sites and 200 μl injected subcutaneously into a rabbit. The animal was boosted six weeks later with 18 μg of purified inhibin, using the same injection procedure as above. The titre of antibody in the rabbit serum was assessed by its ability to bind to iodinated inhibin (for details see below), or by its ability to neutralize inhibin activity in vitro. The highest titre was observed two weeks post booster (week 8 sample), returning to preimmunization levels by 17-18 weeks.

During immunization, the rabbit increased its testicular volume from 3.0 to 3.5 ml, indicating that immunization against inhibin can increase gonadal weight, presumably by neutralization of endogenous inhibin, thus allowing FSH levels to rise.

Example 6: Antiserum Characterization

The week 8 antiserum from the rabbit, prepared as described above, was investigated for its ability to neutralize inhibin activity in vitro. A charcoal-treated bovine follicular fluid preparation was used as inhibin standard in an inhibin in vitro bioassay (Scott et al, Endocrinology, 107, 1980, 1536). It was found that 2 μl of antiserum was sufficient to neutralize a dose of inhibin (2 units) known to give a maximal response in the assay. This neutralizing activity was not present in preimmunization serum. One other rabbit was immunized initially with a less pure inhibin preparation (340 μg obtained after the RP-HPLC purification step) and boosted with 22 μg of pure inhibin.

The initial immunizing injection was in complete Freund's adjuvant, using the immunizing method of Vaitukaitas et al (Journal of Clinical Endocrinology and Metabolism, 33, 1971, 988), while the booster injection procedure was the same cited above. Antiserum (week 9) from this animal also showed neutralizing capabilities in vitro.

Example 7: Radioimmunoassay of bFF Inhibin

Purified preparations of inhibin were iodinated either by a mild chloramine-T procedure or by using the Bolton-Hunter reagent (Bolton and Hunter, Biochem J. 133 (1973) 529) to a specific activity of 0.5 μCi/μg as determined by a self displacement procedure in the radioimmunoassay method described below. The iodinated material showed the same apparent molecular weight as the non-iodinated molecule as assessed by SDS-PAGE under reducing and non-reducing conditions. Using the iodinated tracer, a radioimmunoassay procedure was derived using a polyethylene glycol-facilitated second antibody precipitation step to separate antibody-bound and -unbound iodinated hormone (Peterson, M.A. and Swerdloff, R.S.: Clin. Chem. 25 (1979) 1239-1241). Characteristic displacement curves were obtained for purified and for unfractionated bFF preparations. The dose response curve for the purified material showed a sensitivity (ED_{10}) of 2 ng/tube with ED_{50} of 25 ng/tube. The dissociation constant of the inhibin-antibody interaction was 4.5×10^{-10}M at 20°C.

Example 8: Inhibition of ovulation in human chorionic gonadotrophin-stimulated 5-day pregnant mice

Crude extracts of bovine follicular fluid have previously been shown to inhibit ovulation. The inhibition can be competitively reversed with FSH (L. Cummins, Ph.D.Thesis, 1983, University of New England (Armidale)).

5-7 day pregnant mice were given 1.5 μg inhibin subcutaneously at 9 a.m., followed by subcutaneous injection of 10 IU of HCG at 6.00 p.m. The following morning the number of ova in the ampulla of the Fallopian tube was counted. Inhibin administration significantly inhibited ovulation, as shown in Table 1.

Table 1

Sample	Number of Animals	Number of Ova in Ampulla
Control (Solvent alone)	11	4.91 ± 2.66
Purified Inhibin*	7	2.43 ± 2.22
bFF** (50 μl)	8	3.38 ± 1.80
bFF (100 μl)	4	2.0 ± 0.0

* - Inhibin preparation is 75% pure based on intensity of silver stain on SDS-PAGE. The contaminants consist of higher molecular weight material (M.wt 65-70 kD) which is biologically inactive in the inhibin in vitro bioassay. Dose approximately 1.5 μg protein/animal.

** - Containing 20 μg/ml inhibin based on inhibin in vitro bioassay.

Purified inhibin and 100 μl bFF both resulted in significatn inhibition of ovulation compared to the control ($p < 0.01$ and $p < 0.05$ respectively by Wilcoxon's test).

Example 9: Effect of Immunization on Plasma FSH Levels

In Example 5 above, one rabbit was immunized, and boosted on two further occasions with purified inhibin. The antiserum so obtained neutralized inhibin activity in the in vitro bioassay. It would be expected that in vivo, the

antiserum would neutralize circulating inhibin, leading to an elevation of circulating FSH. The rabbit's plasma FSH showed a rise and fall in concert with the titre of the inhibin antibody in the rabbit serum. The results are shown in Table 2.

Table 2

Weeks Post Booster Injection	Number of Serum Samples	Antibody Titre*	Plasma FSH ng/ml
3-6, 17, 21	6	$\ll 0.25$	4.87 ± 0.76^a
11-14	3	0.67 ± 0.19	5.44 ± 0.24^b
7-10	3	2.13 ± 0.45	6.15 ± 0.44^c

* - Reciprocal of antiserum volume (μl) required to neutralise 1.5 U inhibin in the inhibin in vitro bioassay.

a vs c; a vs b and c; $p < 0.05$

Example 10: Suppression of circulating FSH following acute administration of inhibin to castrated male rats

It is expected that purified inhibin, as seen in the experiment below with bovine follicular fluid, should suppress circulating FSH within 4-8 hours of administration. Inhibin (bovine follicular fluid) was administered via the jugular vein into the circulation of 34-day old male rats which had been castrated 3 days earlier, and the levels of plasma FSH 5 hours later were determined by FSH radioimmunoassay.

There was a significatn dose-dependent decrease in FSH associated with increasing doses of bovine follicular fluid. Results are shown in Table 3.

Table 3

Sample	Number of Animals	Plasma Expressed as % of Pre-injection levels
Control (solvent alone)	5	99.3 ± 12.8[a]
bFF* (62.5 µl)	5	80.8 ± 5.5[b]
bFF (125 µl)	5	66.6 ± 10.3[c]
bFF (250 µl)	5	51.9 ± 7.0[d]

* - containing 20 µg/ml inhibin based on inhibin in vitro bioassay

a vs b; b vs c; c vs d p 0.05

Example 11: Purification of Inhibin From Ovine Follicular Fluid

We have found that inhibin activity from ovine follicular fluid is purified in a similar manner to bFF inhibin using purifications steps A, B and C above. Its characteristics following steps D and E are similar to those of bFF inhibin. By extension it is expected that purification steps A to E would be applicable to other mammalian inhibins including that from human.

Since FSH is important in the stimulation of ovarian and testicular function, the main potential applications of purified inhibin lie in its ability to specifically inhibit FSH secretion, or in its use as an antigen such that immunization against inhibin will elicit antibodies capable of neutralizing endogenously - occurring inhibin, thereby raising FSH levels. Many studies have been performed in vivo using crude or partially purified extracts of gonadal tissues or fluids in attempts to study the action and physiology of

inhibin. In these experiments, effects attributed to but not proven to be due to inhibin or antibodies against inhibin include:

1. Inhibition of gonadal function (Moudgal et al., 1985 in Gonadal Proteins and Peptides and their Biological Significance (ed. Sairam), World Scientific Publishing, Singapore (in press).
2. An increase in ovulation rate (Henderson et al., J. Endocrinol. 102, (1984) 305; Cummins et. al., Proc. Aust. Soc. Reprod. Biol. 15 (1983) 81).
3. An advancement of the onset of puberty (Al Obaidi et al., Proc. Aust. Soc. Reprod. Biol., 15 (1983) 80).

The known properties of inhibin and of FSH suggest a number of possible applications for the purified inhibin and antibody to inhibin according to the present invention:

(i) Increase of ovulation rate: It is recognized that FSH stimulates the development of ova in mammalian ovaries (Ross et. al., (1981) in Textbook of Endocrinology, ed. Williams, p. 355) and that excessive stimulation of the ovaries with FSH will lead to multiple ovulations (Gemzell, Induction of ovulation with human gonadotrophins, Recent Prog. Hormone Res. 21 (1965) 179). We have demonstrated that inhibin will suppress FSH both in vitro and in vivo and that inhibin can be used as an immunogen to raise neutralizing antibodies against inhibin. The immunization of mammals, e.g. cattle and sheep, with the purified preparation of inhibin and a suitable adjuvant leads to the development of antibodies in immunized animals. These antibodies neutralize the animal's own inhibin production, thereby removing the suppressive effect on FSH secretion. The resultant elevation in FSH leads to increased stimulation of follicular development in the ovary with an increase in ovulation rate.

Collection of serum from animals immunized against inhibin also provides an antiserum which can be used for passive immunization of other animals. By this method, the injection of the inhibin antiserum neutralizes the animal's own inhibin and hence leads to an elevation of FSH and the subsequent events in stimulating ovulation. Both the passive and the active methods of immunization may be used to increase ovulation rate.

The potential to use inhibin for active immunization to achieve gonadal growth is illustrated by the increase in testicular size of the rabbit tested during immunization against inhibin (Example 5).

(ii) Inhibition of ovarian and testicular function: The recognized importance of FSH in the stimulation of follicular development in the ovary and sperm production in the testis (Ross et. al., (1981), in Testbook of Endocrinology, ed. Williams, p. 355; Bardin and Paulsen, (1981), The Testes, in Textbook of Endocrinology, ed. Williams, p. 293) supports a potential role for inhibin in the suppression of gonadal function. It is expected that the administration of inhibin will lead to a suppression of ovarian and testicular function and a disruption of fertility. This action of inhibin can be used in males and females of the human, ovine and bovine species and is likely to be applicable to other species.

(iii) Advancement of the onset of fertility: It is recognized that one of the earliest events in the onset of puberty is the rise in FSH levels which leads to ovarian and testicular stimulation (Ross et. al. (1981) in Textbook of Endocrinology, ed. Williams, p. 355; Bardin and Paulsen, (1981), The Testes, in Textbook of Endocrinology, ed. Williams, p. 293). The potential exists that immunization of sexually immature mammals

against inhibin, by active or passive techniques, will lead to a premature onset of puberty with attendant stimulation of ovarian and testicular function.

The lifetime reproductive performance of domestic animals such as cows, sheep and pigs depends upon the age of onset of puberty, the intervals between each conception, and the potential for subsequent ovarian failure with advancing age. Immunization of young animals before puberty with inhibin, either by active or passive immunization, neutralises the animal's own inhibin production and leads to an elevation of FSH. This elevation in FSH levels induces pubertal development at an earlier age than normal by stimulation of the gonad. This method can be used to induce precocious puberty in male or female mammals.

(iv) Suppression of puberty: Since FSH is recognized as a crucial factor in the onset of puberty, administration of inhibin may be used as a means of suppressing puberty in unwanted situations, e.g. precocious pubertal development in humans, or in delaying the onset of puberty.

(v) Inhibin can be used as an immunogen to raise antisera or monoclonal antibodies which can be used to develop radioimmunoassays or enzyme-linked immunoassays to measure inhibin, and to develop immunoadsorbent columns to aid in the purification of inhibin.

(vi) Using the above-described antisera a radioimmunoassay system to measure inhibin has been devised which enables the measurement of inhibin in biological samples (e.g. plasma, serum or urine), which is not possible using the previously known in vitro bioassay system of Scott et al (1980). Inhibin levels in plasma or serum will provide an index of Sertoli cell and granulosa cell function for use in the diagnosis of fertile status.

(vii) It is possible that administration of high doses of inhibin may inhibit the secretion of LH. This would further support the ability of inhibin to suppress ovulation.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A protein, inhibin, characterized in that:

(a) The apparent molecular weight as determined by SDS-PAGE is 56,000 ± 1,000;

(b) The isoelectric point is in the range 6.9-7.3;

(c) The protein can bind specifically to Concanavalin A-Sepharose;

(d) The protein consists of two sub-units separable on reduction as determined by SDS-PAGE, characterized in that:

(i) Their apparent molecular weights are 44,000 ± 3,000 and 14,000 ± 2,000 respectively,

(ii) The isoelectric point of the 44,000 molecular weight sub-unit is in the range 6.0-7.0,

(iii) The N-terminal amino acid sequences of the two sub-units are:

Residue	44 kD Sub-Unit	14 kD Sub-Unit
1	xxx	Tyr
2	Ala	Leu
3	Val	Glu
4	Gly	yyy
5	Gly	Asp
6	Phe	Gly
7	Met	Lys
8	Arg	Val
9	Arg	Asx
10	Gly	Ile
11	Ser	Gln
12	Glu	yyy
13	Pro	Lys
14	Glu	Lys
15	Asp	
16	Gln	

(e) The protein can suppress follicle stimulating hormone but not luteinising hormone, thyroid stimulating hormone or prolactin in an in vitro bioassay system.

(f) The protein can be labelled with radioactive iodine.

2. Analogues, derivatives, fragments, and sub-units of the protein according to Claim 1.

3. A method of induction of antibody directed against inhibin characterized by administering an immunogenic amount of a protein according to Claim 1 or Claim 2 to an animal.

4. A method of raising a monoclonal antibody in vitro or in vivo characterized by the use of a protein according to Claim 1 or Claim 2 as the immunogen.

5. A method of assay for inhibin characterized by the use of an antibody against inhibin made by the method of Claim 3 or Claim 4.

6. A method for constructing oligonucleotide probes for subsequent cloning and expression of inhibin genes using recombinant DNA technology, characterized by the use of the amino acid sequences of the protein sub-units according to Claim 1 or Claim 2.

7. A method for suppressing FSH levels in a mammal, characterized by the use of exogenously administered protein according to Claim 1 or Claim 2.

8. A method of raising FSH levels in a mammal, characterized by administering to that mammal antibody against inhibin made by the method of Claim 3 or Claim 4.

9. A method of increasing the ovulation rate of a female mammal, characterized by administration to that mammal of an exogenous protein according to any one of Claims 3, 4 or 8.

10. A method of increasing spermatogenesis in a male mammal, characterised by administration of an exogenous protein according to any one of Claims 3, 4 or 8.

11. A method of reducing fertility in a male or female mammal characterized by administration of an exogenous protein according to Claim 1 or Claim 2.

12. A method of advancing the onset of puberty in a sexually immature male or female mammal characterized by immunizing said animal with inhibin, according to any one of Claim 3, Claim 4 or Claim 8.

13. A method of delaying the onset of puberty or of suppressing puberty in a male or female mammal characterized by administration of an exogenous protein according to Claim 1 or Claim 2.

14. A method according to Claim 13 for treatment of precocious puberty.

15. A method of induction of antibody to inhibin characterized in that an immunogenic dose of the protein according to Claim 1 or an analogue, derivative, fragment or sub-unit thereof according to Claim 2 is administered to an animal in one or more doses given separately or together.

16. A method according to Claim 15 in which the immunogenic dose is given by intramuscular and/or subcutaneous injection.

17. A method according to Claim 16 in which at least one immunogenic dose is given together with an adjuvant.

18. A method according to Claim 17 in which the adjuvant is oil-based.

19. A method according to Claim 17 in which the adjuvant for at least one injection is a mixture of Marcol 52 and Montanide 888.

20. A method according to Claim 17 in which the adjuvant for at least one injection is complete Freund's adjuvant.

21. Antibody to inhibin prepared by the method of any one of Claims 3, 4 or 15-19.

22. A method for the isolation and purification of inhibin from material of mammalian origin, characterized by subjecting the starting material to the following steps:

(a) One or more gel permeation chromatography steps;

(b) One or more reversed phase high performance liquid chromatography steps;

(c) One or more preparative polyacrylamide gel electrophoresis steps;

(d) Electrophoretic elution of the purified inhibin.

23. A method according to Claim 22, characterized by subjecting the void volume fraction from the first gel permeation chromatography step to a pH precipitation procedure prior to a second gel permeation chromatography step.

24. A method according to Claim 23 in which the pH precipitation is performed using acetic acid.

25. A method according to Claim 22 in which the gel permeation chromatography step is performed using a gel permeation support with high pore volume.

26. A method according to any one of Claims 22-25 in which the eluent is completely volatile.

27. A method according to any one of Claims 22-26 in which the polyacrylamide gel electrophoresis is performed in the presence of sodium dodecyl sulphate.

28. A method according to any one of Claims 22-27 which the reversed phase high performance liquid chromatography step is carried out using chemically bonded N-alkylsilica column packings of particle size in the range 5-100 μ.

29. A method according to Claim 28 in which the particle size is in the range 30-90 μ.

30. A method according to Claim 28 in which the particle size is in the range 10-30 μ.

31. A method according to Claim 28 in which the particle size is in the range 5-10 μ.

32. A method according to any one of Claims 22-31 in which the eluents used in the reversed phase high performance liquid chromatography step are volatile, and contain ionic modifiers selected from the group consisting of perfluoroalkanoic acids, ammonium bicarbonate, and ammonium

acetate, in a gradient of water containing a miscible organic solvent selected from the group consisting of methanol, acetonitrile and isopropanol.

33. A method according to any one of Claims 22-31 in which the eluents used in the reversed phase high performance liquid chromatography step are non-volatile, and contain ionic modifiers selected from the group consisting of alkali metal phosphates and alkaline earth phosphates, in a gradient of water containing a miscible organic solvent selected from the group consisting of methanol, acetonitrile and isopropanol.

34. A method according to any one of Claims 22-31 in which both volatile and non-volatile eluents are used.

35. Therapeutic compositions containing inhibin according to Claim 1 or Claim 2, together with any pharmaceutically-acceptable diluent.

36. Therapeutic compositions containing inhibin according to Claim 1 or Claim 2, together with any pharmaceutically acceptable delayed release composition.

37. Therapeutic or diagnostic compositions containing inhibin substantially as hereinbefore described.

38. Therapeutic or diagnostic compositions containing antibody against inhibin according to any one of Claims 3, 4 or 21 together with any pharmaceutically-acceptable diluent.

39. Therapeutic compositions containing antibody against inhibin according to any one of Claims 3, 4 or 21, together with any pharmaceutically acceptable delayed release composition.

40. Therapeutic or diagnostic compositions containing antibody to inhibin substantially as hereinbefore described.

41. A method for the determination of the fertility status of an animal, characterized in that levels of inhibin in a biological fluid of the animal are measured.

42. A method for suppressing ovulation in an animal, characterized by the administration of sufficiently high doses of inhibin according to Claim 1 or Claim 2 to the animal to suppress LH secretion.

43. Material for an immunoadsorbent column for purification of inhibin, characterized in that antibody to inhibin according to any one of Claims 3, 4 or 21 is bound to a solid, immunologically inert adsorbent material.

44. A method for the isolation of clones expressing all or part of inhibin genes produced using recombinant DNA technology, characterized by the use of an antibody according to any one of Claims 3, 4 or 21.

45. A method for detection of inhibin mRNA in biological samples, characterized by the use of the amino acid sequences of the protein sub-units according to Claim 1 or Claim 2.

46. Antibody to inhibin according to any one of Claims 3, 4 or 21 labelled according to any previously known method.

47. A diagnostic method characterized by the use of a labelled antibody according to Claim 46.

48. A protein according to Claim 1 or Claim 2 labelled according to any previously known method.

49. A radioimmunoassay for inhibin characterized in that a radiolabelled protein according to Claim 48 is reacted with an antibody to inhibin.

50. A radioimmunoassay for inhibin characterized in that a protein according to Claim 1 or Claim 2 is reacted with a radiolabelled antibody according to Claim 46.

51. A radioimmunoassay according to Claim 49 or Claim 50, characterized in that a polyethylene glycol-facilitated second antibody precipitation step is used.

52. An enzyme-linked immunoassay characterized by the use of a protein according to Claim 1 or Claim 2 coupled to an enzyme by any previously known method.

53. An enzyme-linked immunoassay characterized by the use of an antibody according to any one of Claims 3, 4 or 21 coupled to an enzyme by any previously known method.

54. A method for assessing gonadal function characterized by the use of a radioimmunoassay according to any one of Claims 49 to 51 or an enzyme-linked immunoassay according to Claim 52 or Claim 53.

55. A method for the assay of inhibin in a biological sample characterized by the use of a radioimmunoassay according to any one of Claims 49 to 51.

56. A method for the assay of inhibin in a biological sample characterized by the use of an enzyme-linked immunoassay according to Claim 52 or Claim 53.

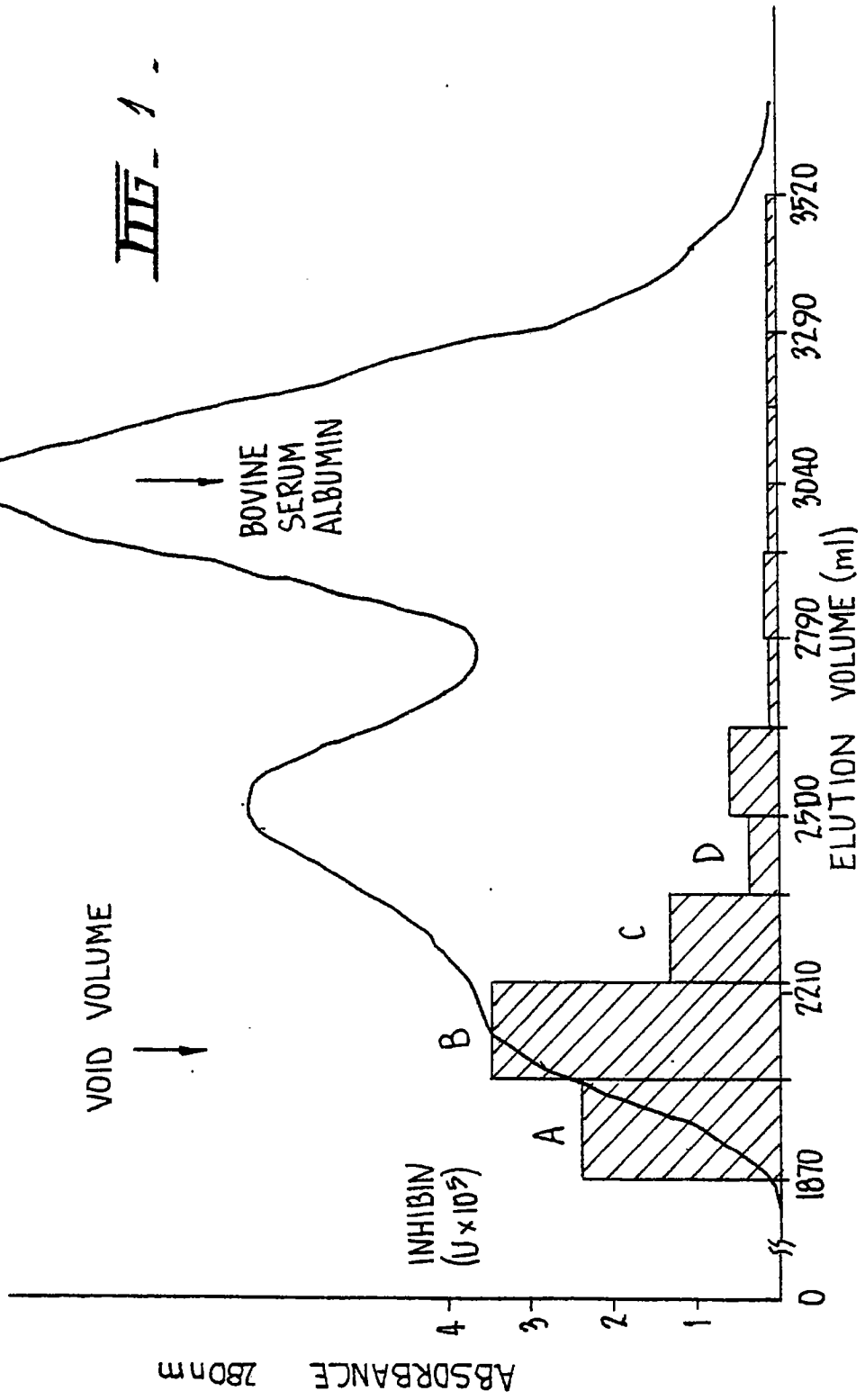
57. A method for immunolocalization of inhibin characterized in that labelled antibody to inhibin according to Claim 46 is used as a marker for inhibin in tissues.

58. A method of suppressing FSH secretion by a tumour, characterized by administration of a protein according to Claim 1 or Claim 2.

59. A method of suppressing inhibin secretion by a tumour, characterized by administration of an antibody according to any one of Claims 3, 4, 21, 38 or 39.

60. A method of raising FSH levels in a mammal characterized by the administration of an exogenous protein according to the method of Claim 3 or Claim 4.

Fig. 1.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1
2
3
4
INHIBIN (U x 10⁵)
VOID VOLUME
BOVINE SERUM ALBUMIN
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B
C
D
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2790
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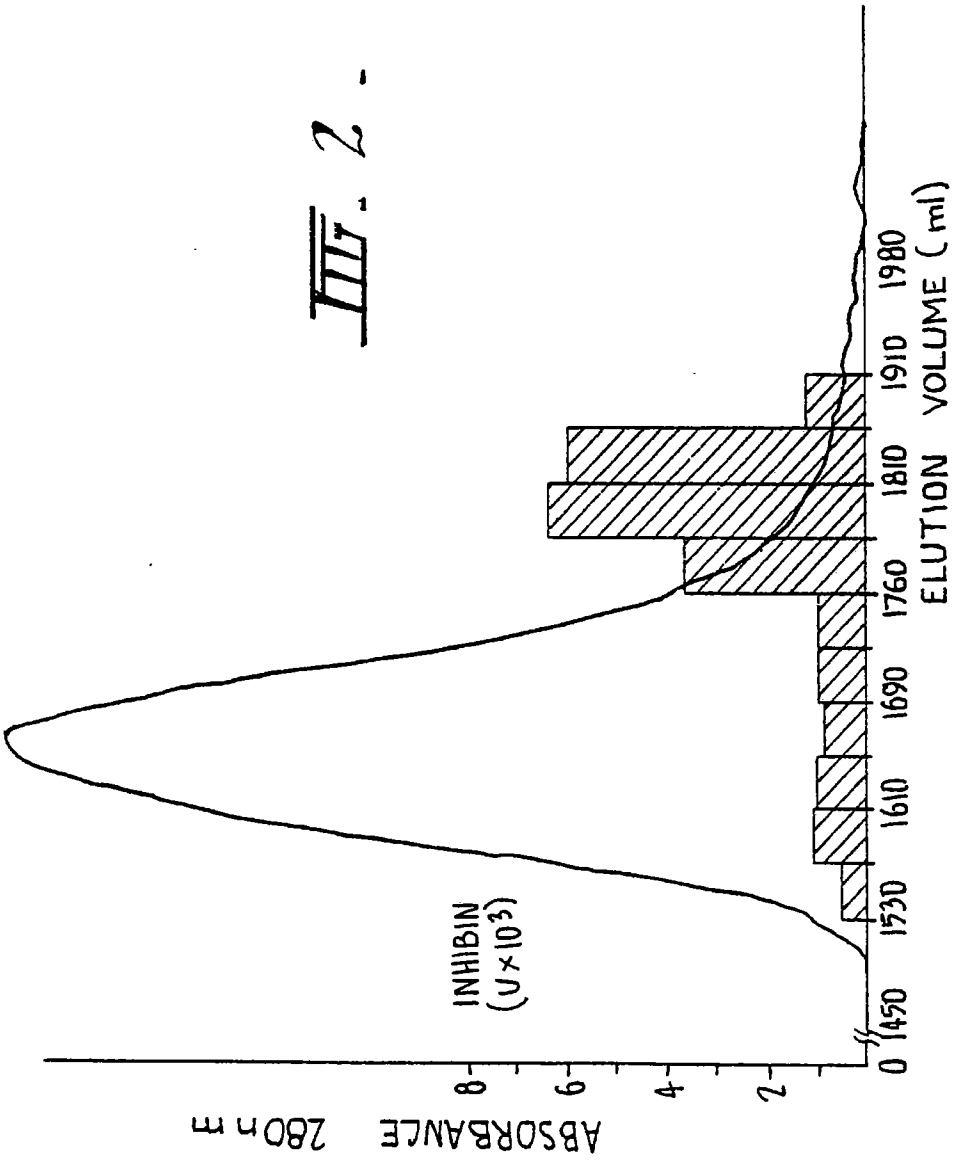
3290
3520
ELUTION VOLUME (ml)

Fig. 2.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6
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1610
1690
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1910
1980
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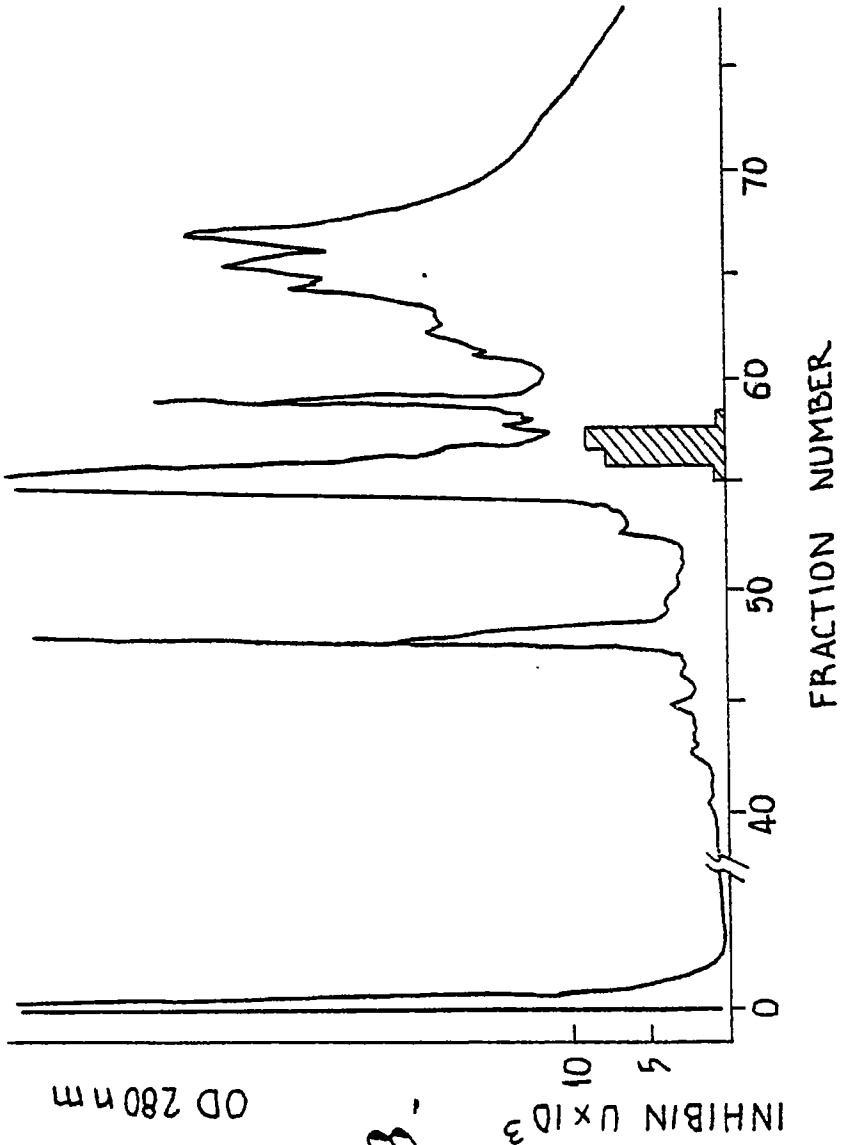

FIG. 3.

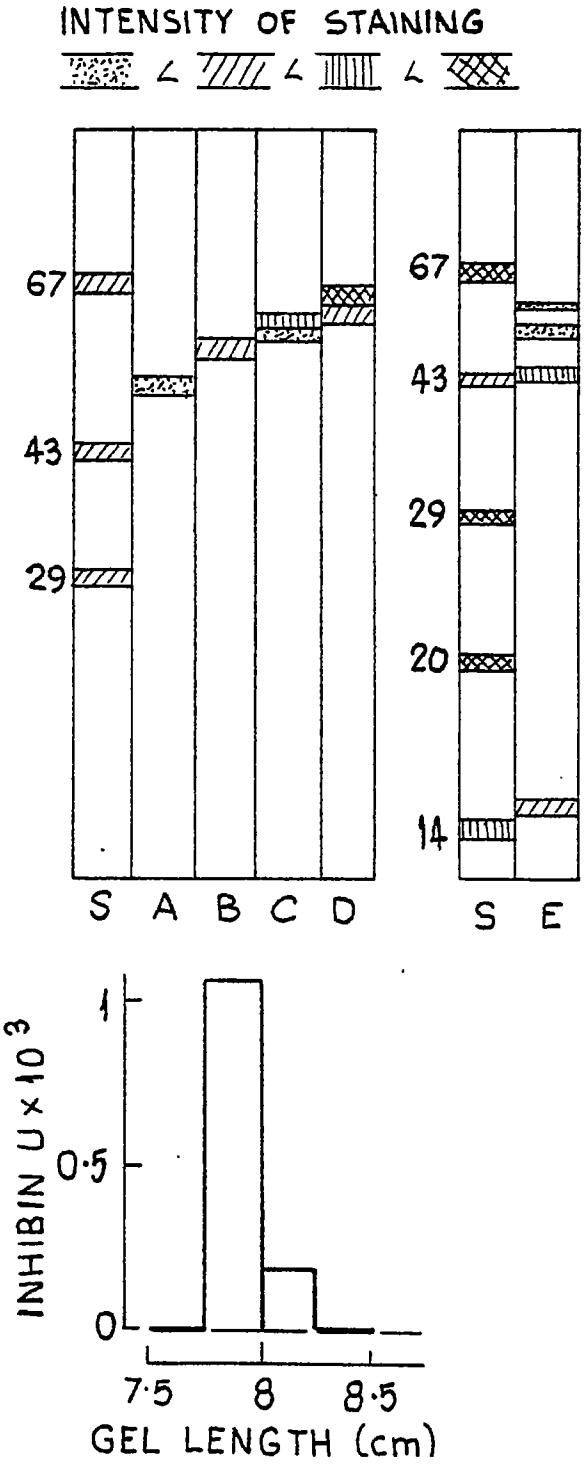
INTENSITY OF STAINING
67
43
29
S A B C D
67
43
29
20
14
S E
INHIBIN U x 10 3
1
0·5
0
7·5 8 8·5
GEL LENGTH (cm)

FIG. 4.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 85/00119

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07K 15/06, 15/14

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C07K 15/06, 15/14
IPC[3]	C07G 7/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
A	Molecular and Cellular Endocrinology, Volume 13, issued 1979 (Elsevier/North-Holland Scientific Publishers), F.H. de Jong, "Inhibin - Fact or Artifact", see pages 1 to 10	1-60
A	FEBS Letters, Volume 167, No.1, issued 1984 February (Elsevier), M. Chrétien et al, "Partial Amino Acid Sequence of a Human Seminal Plasma Peptide with Inhibin-like Activity", see pages 98 to 102	1-60
A	FEBS Letters, Volume 182, No.1, issued 1985 March (Elsevier), H. Lilja et al, "Amino Acid Sequence of the Predominant Basic Protein in Human Seminal Plasma" see pages 181 to 184	1-60
A	FEBS Letters, Volume 165, No.1, issued 1984 January (Elsevier), A.R. Sheth et al, "Characterisation of a Polypeptide from Human Seminal Plasma with Inhibin (Inhibition of FSH secretion)-like Activity", see pages 11 to 15	1-60
A	International Journal of Andrology, Volume 7, issued 1984, (Scriptor, Copenhagen), A.R. Sheth et al, "Evidence for the Prostatic Origin of Immunoreactive Inhibin-like Material in Human Seminal Plasma", see pages 389 to 397	1-60

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 August 1985 (29.08.85)	(06.09.85) 6 SEPTEMBER 1985
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	D.E. Glanville D.E. GLANVILLE

Form PCT/ISA/210 (second sheet) (January 1985)